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A Colorimetric Method for the Determination of Monosaccharides in Organic Solvents for Use in Partition Chromatography

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It is necessary, when using partition chromatography for the separation of substances according to the flowing chromatogram technique, to have some simple identification reaction. It should preferably be directly applicable to the collected fractions and be suitable for mass analyses. Moore and Stein [1] used the ninhydrin reaction for the determination of amino acids quantitatively. For the identification of purine bases Edman, Hammarsten, Löw and Reichard [2] made use of the light absorption at 260 mμ. Both methods can be applied directly to the mobile phase in partition chromatography.

No simple method applicable to sugars has hitherto been described. Bell [3] was the first to separate sugars by means of partition chromatography on a column. He separated methylated monosaccharides on a column of silica gel. Hough, Jones and Wadman [4] later separated unsubstituted sugars on a column of pulverised cellulose. The last-mentioned authors applied the flowing chromatogram technique and made qualitative identification of the sugars by means of paper chromatography.

The usual reduction methods with more or less concentrated salt solutions cannot be used for the determination of sugars in organic solvents. In this case the solvent must at first be removed by evaporation under reduced pressure.

Colorimetric methods which involve the use of strong mineral acids cannot be used either, because the organic solvents are decomposed by the strong acid and give rise to dark-coloured compounds.

The methods using *e.g.* picric acid, dinitro-salicylic acid or o-dinitrobenzene, could possibly be used in the organic solvents.

The picric acid method was found to be applicable only to sugar concentrations greater than 100 μg/ml. The o-dinitro-benzene reaction is very sensitive. It was possible to detect glucose in concentrations as low as approximately 1 μg/ml. The dark-blue colour formed was, however, too unstable.

Aniline phthalate or oxalate has been recommended by Partridge [5] for the qualitative identification of sugars in paper chromatography. Hough, Jones and Wadman [6] have recently studied the salts of a number of bases such as aniline, dimethylaniline, naphthylamine and *p*-anisidine with organic acids such as acetic acid, trichloracetic acid and oxalic acid with regard to their use as spraying reagents in paper chromatography. They found that these salts on heating reacted with aldohexoses and aldopentoses with the formation of coloured compounds. Aldohexoses gave a green to a greenish-brown colour, depending on the sugar and the base used, whereas aldopentoses gave a reddish colour. With these reagents, ketohexoses gave no colour. The authors recommended — as did Forsyth [7] and Partridge [8] — naphthoresorcinol and hydrocloric acid or trichloracetic acid for the identification of ketohexoses. Aniline salts were also found to give coloured compounds with methylated sugars.

It should therefore be possible to use aniline in combination with organic acids for quantitative determinations as well.

Whilst the writer was working on the method described in the following Blass, Macheboeuf and Núñez [9] reported a method for the quantitative determination of sugars. They used Partridge's aniline phthalate reagent. They did not, however, obtain any colour directly till after the reagent was added and the solvent evaporated. After evaporation to dryness the coloured reaction product was taken up in methanol for colorimetry.

On a study of the ability of a number of bases to form colour in combination with various organic acids, the present writer found aniline trichloracetate in a strong solution of trichloracetic acid to give the best results. Colour was formed with aldopentoses and aldohexoses but not with fructose. In the case of both pentoses and hexoses, the colour formed showed the strongest light absorption at 370 mμ. A similar colour with the same absorption maximum was obtained with furfural. The reaction for both categories of sugars and for furfural was found to follow Lambert-Beer's law within the 5—500 μg/ml range. The method was therefore suitable for the quantitative determination of sugars in organic solvents.

EXPERIMENTAL

Reagent: 32 ml of an 8.5 *N* aqueous solution of CCl_3COOH are mixed with approximately 50 ml of cold absolute alcohol in a flask placed in an ice-water bath and 2 ml of aniline are added. After dilution to 100 ml with cold absolute alcohol, the reagent is ready for use.

If alcohol of room temperature is used in preparing the reagent, or if this takes place without chilling, higher blanks are obtained. If the reagent is stored in a refrigerator at approximately 0° C for 3—4 hours, the blank remains constant. After this time a faint yellow colour appears in the reagent and the blank rises simultaneously. The trichloracetic acid to be used for the preparation of the 8.5 *N* solution is kept airtight in a closed bottle in the cold. The 8.5 *N* aqueous solution of trichloracetic acid remains stable for a week.

Sugar solutions: Stock solutions are made in organic solvents saturated with water and containing 1 or 2 mg/ml of the aldopentoses or the aldohexoses. Suitable dilutions can then be prepared from these stock solutions.

Method: 1 ml of the solvent used containing between 5 and 300 μg sugar per ml is pipetted into thin-walled test tubes, 15.4—16 mm by 120 mm, standing in a cooling bath. 1 $\pm$ 0.02 ml of the reagent is added to each test tube. After shaking for 3—5 minutes, the test tubes are placed in a specially constructed stand, provided with a cooling arrangement (see below). The openings of the tubes are covered with aluminium caps and the stand placed in a strongly boiling water bath for 15 $\pm$ 0.5 minutes. After boiling, it is cooled for 5 minutes in a water bath (running tap water or an ice-water bath) and 2 $\pm$ 0.1 ml of 95 per cent alcohol added to each test tube. Reading off takes place in 1 cm cuvettes at 370 mμ in a Beckman spectrophotometer (model B) or a similar apparatus.

At least three blanks are boiled at the same time. The blank consists of 1 ml of solvent saturated with water and 1 ml of the reagent. In chromatography, fractions that do not contain sugar should be used for the blanks. The blanks are compared with a reference solution of the same composition as the blank but that has not been heated in a water bath. The sample is read off against the blank which corresponds most closely to the mean value of the three blanks read off against the reference solution. The blank must not give a higher optical density than 0.070 in a 1 cm cuvette.

Calculations: A series of standard solutions is boiled at the same time as the sample. The series should contain 3 blanks and 3 samples of each of the following solutions: 10, 20, 50, 100 and 200 μg/ml of the sugar to be determined dissolved in the solvent used, saturated with water. On the basis of the optical density of these standard solutions, a standard curve is drawn from which the sugar concentration in the sample is read off directly. When less accuracy is required, it is unnecessary to boil the standard solutions simultaneously each time.

Apparatus: Because the volume of the reagent affects the intensity of the colour (see below), the volume must be kept constant during boiling. It was therefore necessary to construct a stand for the test tubes, provided with a cooling arrangement. It was constructed in such a way, that during boiling, the lower one fourth of each test tube was standing in the boiling water, the middle was rinsed by the cooling water and the upper one fourth was above the surface of the cooling water, the openings of the test tubes being covered with an aluminium cap. The construction of the stand and the water bath is shown in Fig. 1. The level of the boiling water is kept just above the level of the liquid in the test tubes. The water bath is heated by means of five 12 mm Bunsen burners.

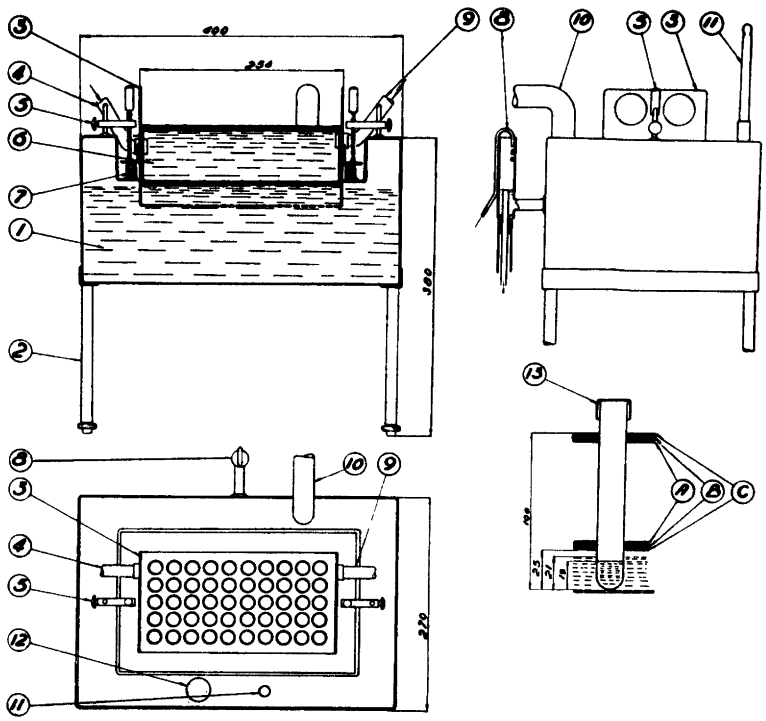

Fig. 1. Test tube stand and water bath.

The stand and the water bath are made of aluminium, thickness 2 mm.

1. *Water bath with boiling water*
2. *Iron stand supporting water bath*
3. *Test tube stand*
4. *Inlet for cooling water*
5. *Screws and clamps for keeping stand in place*
6. *Cooling water in the test tube stand*
7. *Rubber packing*
8. *Water level regulator for water bath*
9. *Outlet for cooling water*
10. *Steam outlet*
11. *Thermometer*
12. *Window for controling of the boiling*
13. *Aluminium cap covering the test tubes*

In order to obtain good tightening of the holes in the stand supporting the test tubes an arrangement shown in detail in the figure was made. A is an aluminium plate with holes 18 mm in diameter (test tube diam. 15.4—16 mm) for the test tubes. B is a rubber plate with holes 14 mm in diameter and C is a plate similar to A.

Factors affecting the formation of colour

1. *Concentration of aniline*: An increase in the concentration of aniline increases the intensity of the colour. That of the blank is increased simultaneously. 2 ml of aniline per 100 ml of reagent resulted in the greatest difference between the blank and the sample, the colour due only to the solvent — *i.e.*, the blank — then being the smallest fraction of the total colour formed.

2. *Concentration of trichloracetic acid*: The concentration of trichloracetic acid has a corresponding effect as that of aniline on the formation of colour, although to a lesser degree.

3. *The degree of purity of the aniline and trichloracetic acid:* The aniline used for preparation of the reagent should be practically colourless. Compared with a somewhat less pure compound, very highly purified aniline causes no improvement. Strongly coloured aniline, on the contrary, gives a high blank. Aniline (A. R.) is distilled *in vacuo* once or twice. Despite repeated vacuum distillations, individual lots of the preparation can nevertheless continue to give high blanks. In such cases, purification according to Keyes and Hildebrand [10] has been found to be satisfactory. The method consists of dissolving the aniline in hydrochloric acid and subsequent removal of the traces of nitro compounds by means of steam distillation. The solution is made alkaline and the aniline distilled with steam. It is then dried over potassium hydroxide and fractionated *in vacuo*.

The trichloracetic acid should be of the highest degree of purity. The optical density of the blank which should not exceed 0.070 in a 1 cm cuvette, is the best gauge of the degree of purity of the aniline and the trichloracetic acid. In the experiments reported here, the trichloracetic acid was obtained in 100 grms lots from A. B. LKB Produkter. It was stored in firmly closed glass vessels in a refrigerator.

4. *Water content*: An increase in the water content of the reagent gives a lower colour intensity and reduces the stability of the reagent. The water content of the reagent is regulated by the quantity of trichloracetic acid solution used and its concentration. An 8.5 N aqueous solution of trichloracetic acid was found to be most suitable.

5. *Volume of the reagent and of the solution*: An increase of $\pm$ 5 per cent in the volume of the sugar solution added causes an inappreciable change in the intensity of the colour formed. The volume of the reagent, on the contrary, has a considerable effect. The reagent should therefore be pipetted off with an accuracy of $\pm$ 0.02 ml.

6. *Duration of boiling and temperature of the water bath:* It is necessary to have a vigorously boiling water bath. If boiling is prolonged, the intensity

of the colour is increased, that of the blank also increasing. Optimal conditions for colorimetry were chosen for the same reason as those given in the case of the concentration of aniline, trichloracetic acid and water.

7. *Effect of dilution after boiling:* If the samples, after boiling, were diluted with different volumes of 95 per cent alcohol, butanol or the reagent the colour did not follow Lambert-Beer's law. The dilution with 2 ml of 95 per cent alcohol after boiling must therefore be made accurately (2 $\pm$ 0.1 ml). If the optical density of a sample is too high for being read off accurately from the apparatus, dilution must take place with a mixture of one part of the solvent in question saturated with water, one part of the reagent and two parts of 95 per cent alcohol. The figure for the sugar concentration obtained from the curve must then be corrected for the dilution.

8. *Influence of amino acids*: When solutions containing 50 μg/ml of glucose and 500 μg/ml of any one of the common amino acids were analysed there was no difference in colour intensity compared with that of solutions containing no amino acid except for cystein. This amino acid caused a detectable depression of colour intensity the amino acid concentration being as low as 50 μg/ml.

Stability of the colour: The change in the intensity of the colour at various times after boiling is shown in Table 1.

Table 1.

Glucose	Optical density after		
μg/ml	15 min.	1 h	24 h
0	0.050	0.050	0.050
10	0.055	0.050	0.055
50	0.260	0.260	0.260
100	0.530	0.515	0.520
200	1.035	1.010	1.010

In these cases, the blank was read off against the reference solution and the other solutions against the blank. Between the readings the solutions were kept in closed test tubes at room temperature.

Absorption spectrum: The light absorption for wave lengths between 320 and 600 mμ is shown in Fig. 2 for hexoses (glucose) and in Fig. 3 for pentoses (ribose). The blank and the standard solutions with 50 and 100 μg/ml were read off against the reference solution, the standard solutions then being read off against the blank. From 355 mμ up to 600 mμ the light absorption of the blank was low and almost constant. Both pentoses and hexoses showed a maximum at 370 mμ.

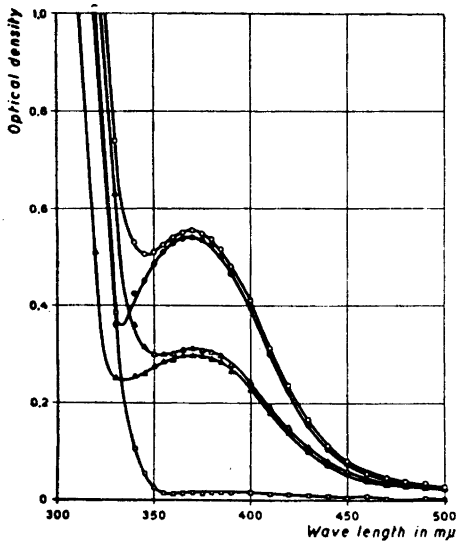

Fig. 2. Absorption spectrum for glucose.

—□—□—□— *Blank read against reference solution*
—△—△—△— *50 μg/ml read against reference solution*
—○—○—○— *100 μg/ml read against reference solution*
—▲—▲—▲— *50 μg/ml read against blank*
—●—●—●— *100 μg/ml read against blank*

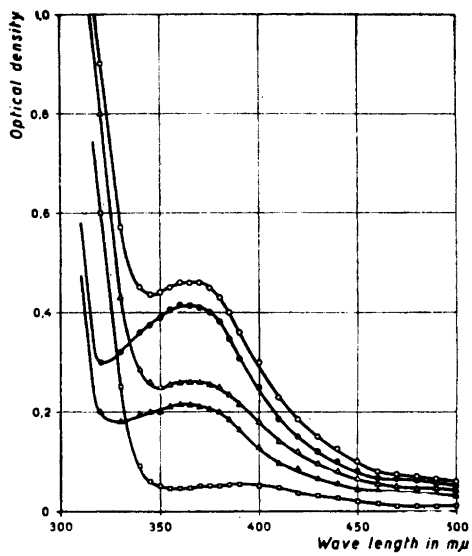

Fig. 3. Absorption spectrum for ribose.

—□—□—□— *Blank read against reference solution*
—△—△—△— *50 μg/ml read against reference solution*
—○—○—○— *100 μg/ml read against reference solution*
—▲—▲—▲— *50 μg/ml read against blank*
—●—●—●— *100 μg/ml read against blank*

Correlation between the sugar concentration and the intensity of colour: The standard curves for glucose and ribose are shown in Fig. 4 and Fig. 5 respectively. It is seen that there is a satisfactory correlation between the colour formed and the sugar concentration in the whole range covered by the curve.

Accuracy: In order to determine the accuracy of the method the following experiment was made.

Three blanks and 10 samples of each of the following solutions: 10, 20, 50 and 100 μg/ml dissolved in butanol saturated with water were boiled simultaneously. A curve was plotted through the means found for the different concentrations from which the sugar content of each individual sample was read off. The mean and the standard deviation of a single determination (σ) were calculated for each solution. The result is shown in Table 2.

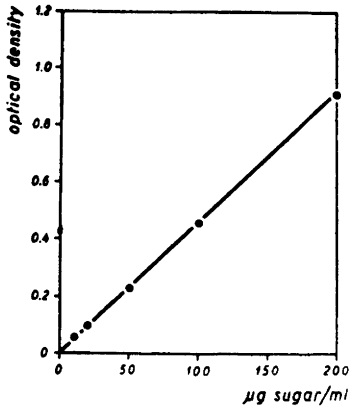

Fig. 4. Standard absorption curve for glucose dissolved in butanol.

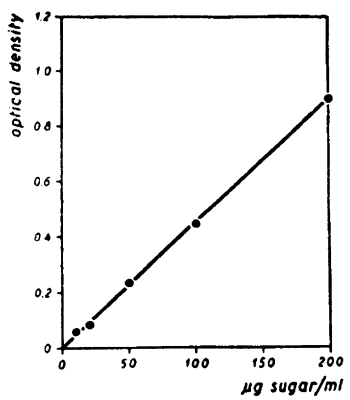

Fig. 5. Standard absorption curve for ribose dissolved in butanol.

Table 2.

Sugar concentration in µg/ml		Standard deviation (σ)	
Added	Found mean	µg	Per cent of the mean
10	10.1	1.9	19
20	18.75 (!)	1.25	6.7
50	49.5	1.20	2.4
100	100.7	2.65	2.7

Applicability to different solvents: The method was applied to mixtures of butanol and ethanol and of butanol and propanol. High blanks were obtained in ethylene glycol and in ethyl acetate.

SUMMARY

A method is described for the determination of monosaccharides in organic solvents to be used in partition chromatography. It is based on a reaction between aniline salts and sugar

The method can be used for aldopentoses, methyl pentoses and aldohexoses in concentrations between 10 and 300 µg/ml.

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